Somatic Embryogenesis from Embryogenic Leaf Callus of Tea (*Camellia sinensis* (L.) Kuntze)

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ABSTRACT. *The aim of this study was to produce somatic embryos indirectly from embryogenic leaf callus of tea (Camellia sinensis (L.) Kuntze). Primary embryogenic calli (friable calli, 16 weeks after culture of in vitro leaf segments) were first cultured on Murashige and Skoog (MS) medium containing Benzyl Aminopurine (BAP) (3 mg/l) and Napthalene Acitic Acid (NAA) (0.1 mg/l) and maintained for 4 weeks. Primary calli were then transferred to half and full strength MS media containing BAP and NAA in combination with Abscisic Acid (ABA) (0-1.0 mg/l) to select the suitable second medium. MS medium supplemented with BAP (1.0 mg/l) and NAA (0.1 mg/l) was found to be the second best medium for somatic embryogenesis. Further work was done to select the optimum culture duration for maintaining primary calli on first and second media for efficient somatic embryogenesis. It was noted that the production of somatic embryos was relatively high (8.3%), but the size of embryos was very small (1 mm long) when the primary calli were kept for 8 weeks on the second medium after maintaining them on the first medium for 8 weeks. Meanwhile, 2 mm long somatic embryos were obtained from the primary calli cultured directly on the second medium without maintaining them on the first medium. Rates of somatic embryogenesis were not significant in both patterns of culture periods. Protocol developed on indirect somatic embryogenesis will be useful in order to achieve new somatic variants from seedling explants and also to use in transformation work.*

INTRODUCTION

During the past 20-30 years, significant progress has been made in *in vitro* propagation of tea (*Camellia sinensis* (L.) Kuntze) cultivars directly or indirectly through organogenesis (Gunasekare and Evans, 2000a; Sarathchandra *et al*., 1997) and somatic embryogenesis (Akula and Akula, 1999; Akula *et al*., 2000; Bag *et al*., 1997; Mondal *et al.,* 2000; Ponsamuel *et al*., 1996; Wachira and Ogada, 1995) to produce large number of plantlets in a relatively short period of time. *In vitro* propagation technique via organogenesis is a two-step process for the growth of shoots and roots. Somatic embryogenesis on the other hand, is a one-step procedure to form bipolar structure and hence this system will save labor, time, space and money in production of plants. An important factor in tea that limits the commercial exploitation of micropropagation technique is the rapid loss of juvenility in cultures, which leads to lower multiplication rate.

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In addition, the resulting plants will only have adventitious roots and hence are highly susceptible to drought (Dodd, 1994).

New elite tea cultivars have been mainly developed by selection. However, it is possible to produce elite material within the existing cultivars. Improvement of tea depends on the presence of adequate genetic variability. Therefore, it is necessary to increase genetic variability. Theoretically, indirect regeneration gives rise to plants with genetic variability. Shoot organogenesis via callus seems to be relatively difficult in *Camellia* species, especially from the vegetative parts (Frish and Camper, 1987; Gunasekare and Evans, 2000b; Wu *et al*., 1981). Therefore, somatic embryogenesis could be applied to generate somaclonal variants. Thus *in vitro* screening is possible in order to select few desirable cultivars. Further, somatic embryos with genotypes of selected elite parents are a potential source in the production of artificial seeds, for germplasm storage, genetic transformation and also for genetic and biochemical studies. Somatic embryogenesis in tissue culture has wide biotechnological applications by virtue of its potential to bring about widespread changes (Lutz *et al*., 1986; Tulecke, 1987).

Of these reports, Kato (1996) and Sarathchandra *et al*. (1988) used leaves as initial explants in indirect somatic embryogenesis, where only Kato (1996) achieved a significant success in inducing somatic embryos either directly or indirectly from leaf explants cultured in the presence of various levels of 2, 4 D incorporated on MS medium. However, only certain genotypes can respond to 2, 4 D by initiating somatic embryos from leaves in tea plants. Further, treatment with NAA was more effective than 2, 4 D when used alone or in combination with BAP for embryogenic callus and somatic embryo induction from seeds of *Eschscholzia californica* (Park and Facchini, 1999). Therefore, this research was aimed at developing a protocol for efficient somatic embryogenesis from embryogenic leaf callus of tea.

MATERIALS AND METHODS

Production of primary callus from *in vitro* leaves

Leaf segments (5 mm × 5 mm) were excised from *in vitro* micro shoots of tea cultivar TRI 2043 and cultured on callus induction medium [MS (Mrashige and Skoog, 1962) basal medium with Benzyl Aminopurine (BAP) (2 mg/l), Napthalene Acitic Acid (NAA) (3 mg/l) and sucrose (3.0% w/v); solidified with 0.8% (w/v) agar]. Culture bottles (125 ml capacity), each containing 10 ml medium and six explants, were incubated at 22 ± 2^0C under white fluorescent light (16 h photoperiod, 25 μmoles/m^2/s). At the 8^{th} week of incubation, explants forming callus were transferred to similar fresh medium but with 0.7% (w/v) agar, for proliferation of embryogenic calli.

Effect of the strength of MS basal medium and ABA on somatic embryogenesis

Primary calli (friable calli, 16 weeks after culture of leaf segments) were placed on the culture vials (28 ml capacity) for the induction of somatic embryoids. Primary calli were maintained on first differentiation medium [abbreviated as DM1 medium: MS basal medium with BAP (3.0 mg/l), NAA (0.1 mg/l) plus 3.0% (w/v) sucrose and solidified with 0.7% (w/v) agar] for 4 weeks and were then transferred to different media [each with 2.0% (w/v)

sucrose and 0.8% (w/v) agar] as shown in Table 1, to select the suitable second differentiation medium for the development of somatic embryos. Calli were transferred to fresh media every 8 weeks. Two independent experiments were conducted. Each experiment had 24 samples (primary calli) per treatment.

Table 1. Culture media tested for the formation of somatic embryos from embryogenic callus of *Camellia sinensis* (L.) Kuntze.

Media Code	Composition of media
SM1	1. Half strength MS basal medium devoid of growth regulators.
SM2	2. Half strength MS basal medium + 1 mg/l BAP + 0.1 mg/l NAA.
SM3	3. Half strength MS basal medium + 1 mg/l BAP + 0.1 mg/l NAA + 0.1 mg/l ABA.
SM4	4. Half strength MS basal medium + 1 mg/l BAP + 0.1 mg/l NAA + 0.5 mg/l ABA
SM5	5. Half strength MS basal medium + 1 mg/l BAP + 0.1 mg/l NAA + 1.0 mg/l ABA
DM1	6. Full strength MS basal medium + 3 mg/l BAP + 0.1 mg/l NAA
DM2	7. Full strength MS basal medium + 1 mg/l BAP + 0.1 mg/l NAA

Morphological and histological studies on somatic embrogenesis were carried out. For cytological examination, embryonic calli were fixed in fixation [70% alcohol: 40% formaldehyde: acetic acid (glacial) at 17:2:1 (v/v)]. The fixed samples were then dehydrated in a graded ethanol series and embedded in paraffin wax (Sigma) as described by Harris *et al*. (1994). The embedded tissues were cut into 6-7 µm thick sections using a semi automatic rotary microtome (type cut 5062/6062) and the longitudinal sections were stained with totuidene blue (0.05% w/v) and dissolved in 0.5% (w/v) aqueous sodium tetraborate for 2 min. prior to microscopic observations.

Effect of culture duration on somatic embryogenesis

Primary callus (12 or 16 weeks after culture initiation of leaf segments on callus induction medium) developed from each leaf segment was cut into four small pieces (uniform size). They were cultured on the DM1 medium (two explants/vial). Each culture vial (28 ml capacity) consisted of 5 ml of the medium. Subsequently, these callus pieces were transferred to DM2 medium [MS basal medium with BAP (1.0 mg/l), NAA (0.1 mg/l), 2.0% (w/v) sucrose and solidified with 0.8% (w/v) agar] that was selected from the previous experiment. Sub culturing period to fresh medium was 8-weeks. Each treatment consisted of 36 callus pieces. This experiment was repeated once. Morphogenic responses (nodule and globular structure formations) in terms of somatic embryo formation were observed on the production of somatic embryos at weekly intervals. Details of culture intervals need for the experiment are given in Table 2.

Statistical analysis

Wherever possible, data were analyzed using the SAS software. The percentage data were first subjected to Arc-sine transformation before analysis of variance. The

significant difference between means was estimated at the 5% level using Duncan's Multiple Range Test.

Table 2. Culture intervals used for somatic embryogenesis of *Camellia sinensis* (L.) Kuntze.

Code	*Culture period (weeks)		
	Callus medium	DM1 medium	DM2 medium
L1	12	0	20
L2	12	4	16
L3	16	0	16
L4	16	4	12
L5	16	8	8
L6	16	16	0

Note: *Total culture length is 32 weeks.
Callus medium: MS basal medium + 2 mg/l BAP + 3 mg/l NAA.
DM1 medium: MS basal medium + 3 mg/l BAP + 0.1 mg/l NAA.
DM2 medium: MS basal medium + 1 mg/l BAP + 0.1 mg/l NAA.

RESULTS AND DISCUSSION

When primary calli were maintained on DM1 medium (1st differentiation medium) for four weeks, globular, heart-shaped somatic embryos (1-2/explant) were observed in a few calli. Heart-shaped embryo is a typical embryo *i.e.* bipolar structure having two cotyledon-primordia. These embryos developed gradually and formed mature somatic embryos. Distinct stages in somatic embryogenesis required sequential changes in the medium composition, especially the growth regulators (Ammirato, 1986).

According to the histological studies, embryogenic cells differentiated to 3 to 4 celled proembryoids after 16 weeks of incubation. Cambium - like zones were seen in the transverse section of nodule, which arose from calli and had a broad contact with maternal tissue (Fig. 1A). The sequences of cell division gave rise to somatic embryoids that consisted of two distinct poles (Figs 1B, C and D). The embryogenic cells, from which embryiods visibly derived, show a number of common features (small cell with a large nucleus, dense cytoplasm and small vacuoles), which are characteristic of meristematic cells (Williams and Maheswaran, 1986).

Nodular formations (round, finger-like structures) were observed in calli during the first two weeks after culturing on different media (2nd differentiation media) as shown in 2 Table 1. However, the most friable calli on half MS media turned compact reddish or dark green (Figs 2A and B) and gradually died during the 10th-16th week maintained on these 2nd differentiation media. Further, mucilaginous white substances were observed on the surface of calli cultured on half MS medium containing ABA at 0.5 mg/l or 1.0 mg/l during the early culture periods. ABA, which is regarded as a growth regulator, is not often used in tissue culture but has its specific applications such as stimulating embryoid development

from callus (Khanna, 1999). Embryo proliferation from proembryogenic masses obtained from cotyledonary slices of tea increased in half MS liquid supplemented with 2.0 mg/l BAP; their development and maturations were enhanced by the presence of both kinetin and ABA at 0.1 mg/l each using the temporary immersion system (Tahardi *et al*., 2000).

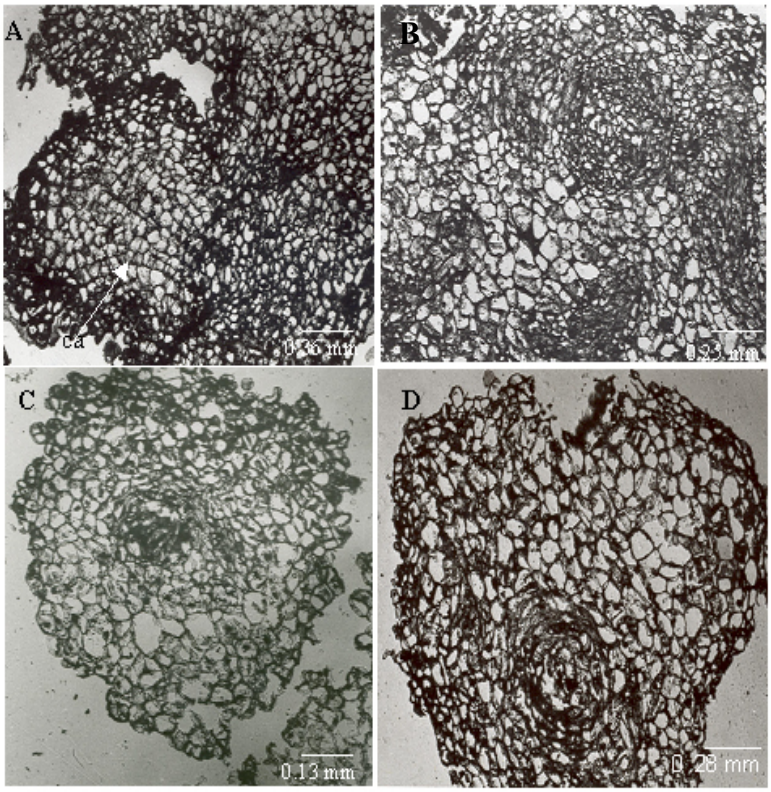

Fig. 1. Histology of somatic embryogenesis from primary callus.

Note: A-B: Transverse sections of primary callus after two weeks maintained on 1st differentiation medium; C-D: Transverse sections of early stages of somatic embryos after maintaining on 1st differentiation medium (C - globular shape; D - Heart shape).

In the present study, among the media tested, yellowish translucent nodules (Fig. 2C) appeared on the surface of the calli cultured on MS medium containing BAP (1.0 mg/l) and NAA (0.1 mg/l) even after 8 weeks maintained on the same medium. In embryogenic cultures, each proembryonic group of cells becomes separated from surrounding cells by thickened, cutinized walls on the outer surface (Williams and Maheswaran, 1986).

The frequency of primary calli that induced numerous small (0.5-1.0 mm diameter) early embryoids (visible to naked eye) was relatively high (37.5%; Table 3) on DM2 medium at 16th week. Very small somatic embryos (1-2 mm long; two embryos per primary callus) were formed. Formation of somatic embryos of various developmental stages of

somatic embryos on the same medium (Figs 2F, G, H, and I) was confirmed by microscopic observations.

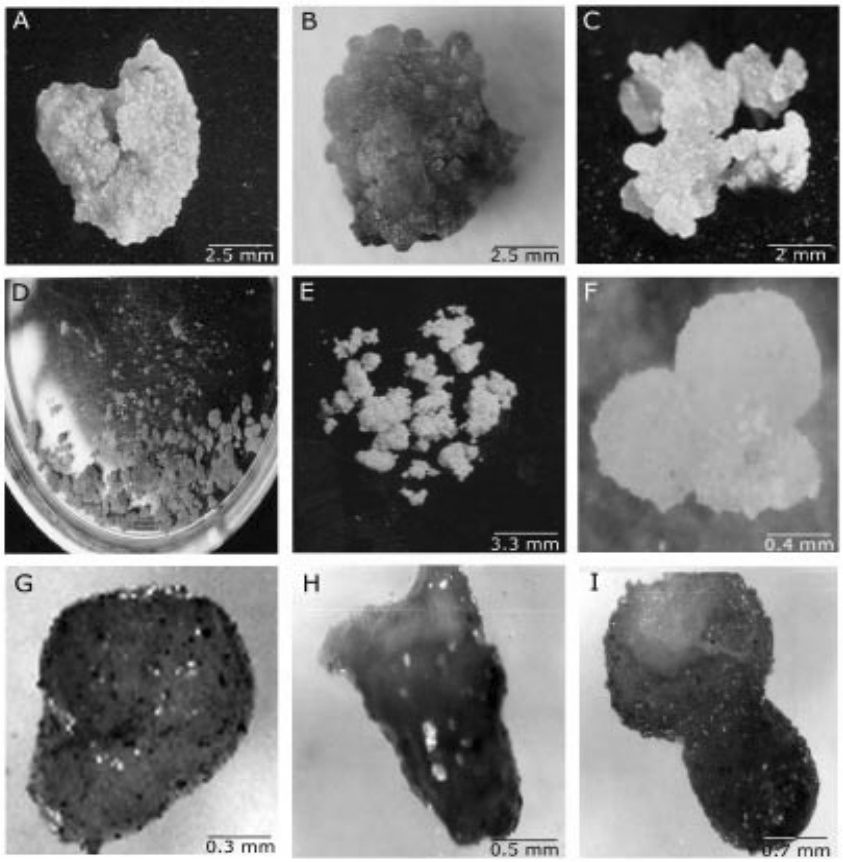

Fig. 2. Somatic embryogenesis from primary callus.

Note: A-C: Primary calli (A, B and C) maintained on 2nd differentiation media (SM2, SM3 and DM2 media) for 8 weeks after maintaining on 1st medium respectively (SM2 - half MS medium with BAP and NAA; SM3 - half MS medium containing ABA at 0.1 mg/l, BAP and NAA; DM2 - MS medium with BAP and NAA); D-E: Primary calli (D and E) maintained in L5 and L6 cultured periods (32 weeks - total culture period after culturing leaf explants); F-I: Various developmental stages of somatic embryogenesis obtained from prmary calli maintained in L4 culture period.

The type of cultured primary calli on DM1 and DM2 media at different culture intervals and also size of somatic embryos (early - late stage) obtained from these primary calli varied among tested treatments. DM1 and DM2 media are 1st and 2nd differentiation media for somatic embryogenesis respectively. The time of transfer of primary calli to differentiation medium is crucial for somatic embryogenesis. Leaf explants cultured on medium with 2, 4 D for shorter (4 weeks) or longer time periods (12 weeks) only formed non-embryogenic callus (Kato, 1996).

Table 3. Effect of the strength of MS medium and ABA on somatic embrogenesis from primary calli.

Medium	At 8th week		At 16th week	
	Survival (%)	Nodule formations (%)	Survival (%)	Early embryiod formations (%)
SM1	70.8[c]	16.7[e]	02.1[c]	00.0[*]
SM2	91.7[ab]	62.5[a]	91.7[a]	18.7[ab]
SM3	95.8[ab]	31.2[cd]	93.7[a]	08.3[b]
SM4	85.4[bc]	50.0[bc]	35.4[b]	00.0[*]
SM5	75.0[c]	16.7[de]	10.4[c]	00.0[*]
DM2	100.0[a]	83.3[a]	100.0[a]	37.5[a]

Note: Values represent the means of two independent experiments, each with 24 primary calli. Means followed by identical letter in each column are not significantly different at $P = 0.05$ according to DMRT test; [*] Data were not analyzed because of zero values.
SM1 [Half MS medium devoid of growth regulators]; SM2 [Half MS + 1 mg/l BAP + 0.1 mg/l NAA]; SM3 [SM2 + 0.1 mg/l ABA]; SM4 [SM2 + 0.5 mg/l ABA]; SM5 [SM2 + 1 mg/l ABA]; DM2 [MS medium + 1 mg/l BAP + 0.1 mg/l NAA].

In L1 and L2 culture periods, the most primary calli gradually changed to compact callus where round nodular structures were noted, but in L6 period, texture of primary calli was yellowish, friable and numerous globules (0.5 mm diameter) were exhibited (Fig. 2E). Even though a high rate (8.3%) of somatic embryogenesis was achieved in L5 period (Fig. 2 D), size of embryos was very small (1 mm long). In L3 period, embryos (2 mm long) were obtained, but no significant result was recorded on the frequencies of somatic embryo formations in these two culture periods (Table 4). Kato (1988; 1996) reported that somatic embryogenesis from leaf explants occurred in the presence of BAP and IBA or with 2, 4 D. In contrast to the present studies, Sarachchandra *et al*. (1988) mentioned that embryoid - like structures were formed in the callus derived from nodal explants but not in the leaf callus of Sri Lankan tea cultivar TRI 2025 where calli were formed from the explants in a combination of 2, 4 D and Kinetin. In the present study, however, considerable amount of somatic embryos (1-2 mm long) were produced in the presence of BAP and NAA.

Table 4. Effect of culture period on somatic embryogenesis from primary leaf calli of *Camellia sinensis* (L.) Kuntze.

Culture periods (weeks)[1] Code	Somatic embryogenesis[2] (%)
L1	1.4[b]
L2	[*]0.0
L3	3.8[ab]
L4	6.9[a]
L5	8.3[a]
L6	[*]0.0

Note: Values represent the means of two independent experiments, each with 36 calli; [1]Data were recorded at 32 weeks after inoculation of leaf segments on callus medium; [2]Formation of somatic embryos at late stages, based on visual observations; [*]Values were not analyzed due to zero values; Means followed by identical letter in each column are not significantly different at $P = 0.05$ according to DMRT test.

CONCLUSIONS

Somatic embryos were developed from embryogenic calli obtained from leaves of *in vitro* shoots, which were established from seed material. In tea plants, seeds are heterozygous in nature and hence further work is necessary to improve the efficiency of somatic embryos and to test the genetic uniformity and quality of embryos produced. Theoretically, somatic embryogenesis with intervening callus stage has genetic variability. Therefore, it provides a new selection method to produce new breeding lines of tea.

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